ORIGINAL

Histochemical and immunohistochemical investigation of guanase and nedasin in human tissues

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Abstract : Guanase is known as an enzyme released from the liver. Recently, cloning and sequencing of the guanase gene were reported. In addition, almost simultaneously, it was reported that an unknown protein that binds to neuronal and endocrine lethal(1)-discs large (NE-dlg), one of the membrane-associated guanylate kinase homologues (MAGUK) family proteins involved in synaptic connection between neurons, was cloned and named nedasin (NE-dlg associated protein), whose sequence was almost identical to that of guanase. We immunostained fresh frozen sections of surgically removed human liver, kidney, and small intestine with anti-nedasin antibody, and simultaneously performed histochemical staining for guanase for comparison. Histochemically, guanase activity was observed in the cytoplasm of hepatocytes and biliary epithelium on the liver, in the mucosal epithelium on the small intestine, and in the proximal tubule on the kidney. Immunohistochemically, a brown discoloration due to DAB oxidation was seen in the cytoplasm of hepatocytes and biliary epithelium on the liver, in the proximal tubule but in the distal tubule a little on the kidney, in the mucosal epithelium on the small intestine. The stained region of the liver and the small intestine were different from that of the kidney. The different staining properties dependent on the organs were considered to be due to different isozymes. The physiological significance of guanase may vary with the isozymes, further studies are considered necessary. J. Med. Invest. 53:264-270, August, 2006

Keywords **:** *guanase, nedasin, histochemical staining, immunohistochemical staining*

INTRODUCTION

Guanase is an enzyme discovered in domestic rabbit liver homogenate by Schmidt (1), and is a deaminase involved in the conversion of guanine to xanthine. Xanthine is further degraded into uric acid by xanthine oxidase. Passanenti (2) discovered that serum guanase activity was increased in patients with liver disease. Then, many investigators (3-7)studied the clinical significance of measuring serum guanase activity, and indicated its usefulness in liver function tests. Human guanase is abundant in the liver, brain, and kidney, but is scarcely contained in the skeletal muscle, heart muscles, pancreas, and others, in which aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are relatively abundant ; thus, an increase of serum guanase activity is considered specific to liver diseases. Ito *et al*. (8-12) reported the local-

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ized distribution of guanase to the cytoplasm of hepatocytes around the human hepatic portal vein, human renal proximal tubule, and mucosal epithelium of the human small intestine by histochemical and immunohistochemical staining, but its functional significance remains poorly understood.

Recent advances in molecular biology have revealed the sequence of the guanase gene and launched new studies. Yuan *et al*. (13) cloned the guanase gene and reported its DNA sequence. Almost simultaneously, Kuwahara *et al*. (14) reported that an unknown protein that binds to neuronal and endocrine lethal(1)-discs large (NE-dlg), one of the membrane-associated guranylate kinase homologues (MAGUK) family proteins that are considered to play various important roles in the adhesion of epithelial cells and synaptic connection between neurons, was cloned and named nedasin (NE-dlg associated protein), whose sequence was identical to that of guanase. However, there is no report concerning the comparison between images of immunohistological staining using anti-nedasin antibody and images of histochemical staining for guanase. Thus, comparison between these staining images is considered useful to reveal the physiological significance of guanase and its functional analysis.

Here, the authors prepared fresh frozen sections of surgically removed human tissues to perform immunohistochemical staining with anti-nedasin antibody and histochemical staining for guanase simultaneously for comparison, and examined the similarities and differences between their staining properties.

MATERIAL AND METHODS

Materials

Reagents

(1) The histochemical staining method

Guanine, N-bis(2-hydroxyethyl)glycine (bicine), xanthine oxidase (XOD)(grade Ⅲ, 19.8 U/ml), and nitro blue tetrazolium (NBT) were purchased from Sigma Chemical Co., U. S. A. Other reagents of the highest available purity were obtained from standard sources.

0.1M Bicine buffer, NBT solution, substrate stock solution, substrate solution, and substrate mixture were prepared as previously reported (8).

(2) The immunohistochemical staining method

An polyclonal antibody against the C-terminal region of nedasin (S and V1 forms) was raised by the immunization of a rabbit (provided by Prof. Hideyuki Saya, Department of Tumor Genetics and Biology, Kumamoto University School of Medicine) was used as a primary antibody in immunohistochemical staining for nedasin; the Dako catalyzed signal amplification (CSA) system (Dakocytomation) was used as the staining kit. Other reagents of the highest available purity were obtained from standard sources.

Tissues

Healthy tissues collected from surgically removed human liver (metastatic liver cancer), kidney (renal cell carcinoma), and small intestine (the jejunum of a patient with resected gastric cancer) were used. Skeletal muscle (striated muscle) was used as a negative control ; human liver with guanase activity was used as a positive control. Our study plan was approved by the medical ethics committee of The University of Tokushima Graduate School.

Methods

(1)The histochemical staining method for guanase (activity staining)

Each tissue was snap frozen in -80℃ isopentan immediately after collection, and 10 μm sections were prepared using a cryostat and were put onto glass slides. These sections were fixed with 2.5% glutaraldehyde solution in cacodylate buffer (0.2M, pH7.4) for 30 min at room temperature, and were washed with 0.1M bicine buffer.

After washing, they were immersed in XOD solution (0.1 mL/10 mL buffer) for 15 min to degrade endogenous xanthine and hypoxanthine. Subsequently, after being washed with bicine buffer and immersed in substrate mixture for 2 hours, they were dehydrated with alcohol and immersed in xylene to be embedded.

As a control experiment, activity staining for guanase was performed simultaneously on human striated muscle lacking guanase activity and human liver with guanase activity. In addition, hematoxyline and eosin (H&E) staining was performed on each adjacent section.

(2) The immunohistochemical staining method for nedasin

Immunohistochemical staining was performed on the adjacent sections with anti-nedasin antibody as a primary antibody using a CSA staining kit.

After treatment with fixing solution (formalin concentrate solution:methanol=1:4) for 20 sec, the sections were immersed in methanol concentrate solution containing 0.3% hydrogen peroxide solution for 30 min to block endogenous peroxidase, and were each washed 3 times with Tris Buffered Saline (TBS) for 5 min. To block non-specific proteins, casein-containing non-specific reaction blocking reagent was dripped onto the sections for 5 min, and after 1 : 1,000 diluted anti-nedasin antibody was dripped for 15 min without washing, they were each washed 3 times with Tris Buffered Saline containing Tween 20(TBST) for 5 min. For the reaction with a biotin-labeled secondary antibody, a biotin-labeled anti-rabbit immunoglobulin antibody was dripped onto the sections for 15 min, and they were each washed 3 times with TBST for 5 min. In addition, streptavidin-biotin complex was dripped onto the sections for 15 min, and they were each washed 3 times with TBST for 5 min. Then, biotin-labeled tyramide was dripped onto the sections as an amplification reagent for 15 min, and after washing with TBST for 5min 3times, peroxidase-labeled streptavidin was dripped for 15 min, followed by washing 3 times with TBST for 5 min.

The coloring reaction was stopped at the appropriate time by observing the brownish coloring of 3, 3'- diaminobenzidine tetrahydrochloride (DAB) with the naked eye and under a microscope while dripping DAB substrate solution onto the sections, and after the coloring was stopped by immersing the sections in distilled water, stained briefly with hematoxylin, they were dehydrated with alcohol and immersed in xylene to be embedded.

As a control experiment, frozen sections of human striated muscle (9), presumed to lack guanase activity, were simultaneously stained.

RESULTS

1. Staining images of guanase and nedasin in each tissue

Figure 1 shows the images of histochemical staining for guanase (a,d), immunohistochemical staining for nedasin (b,e), and H&E staining (c,f) in the human normal liver. Guanase activity was observed in the cytoplasm of hepatocytes and biliary epithelium, but not in the portal components such as infiltrating cells, fibrous tissue, veins, and biliary

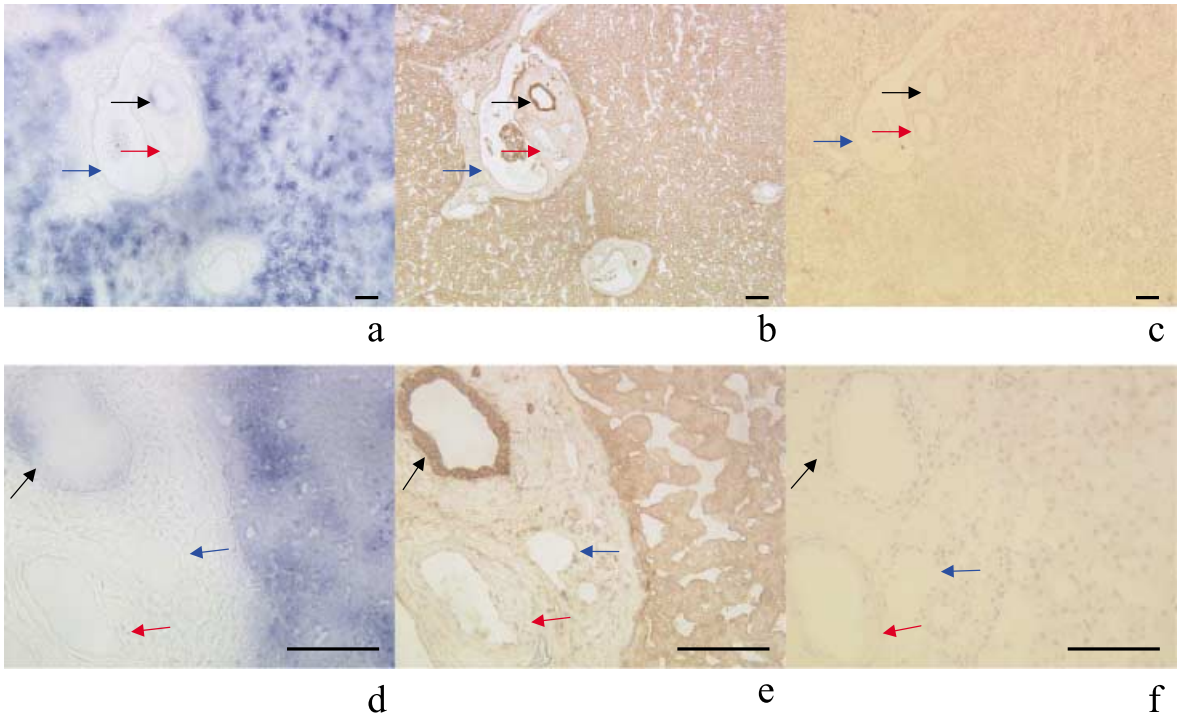

Figure 1 : Histochemical staining for guanase on the frozen sections of the human normal liver (a:×100, d:×400) : Guanase activity was observed in the cytoplasm of hepatocytes and biliary epithelium, but not in the portal components.
Immunohistochemical staining for nedasin on the adjacent sections(b:×100, e:×400) : a brown discoloration due to DAB oxidation was seen in the cytoplasm of hepatocytes and biliary epithelium, but not in the portal components.
H&E staining on the adjacent sections(c:×100, f:×400).

duct (excluding mucosal epithelium). Also, in the immunohistochemical staining for nedasin, a brown discoloration due to DAB oxidation was seen in the cytoplasm of hepatocytes and biliary epithelium, but not in the portal components ; thus, consistent staining results were obtained between the histochemical staining for guanase and the immunohistochemical staining for nedasin.

Figure 2 shows the images of histochemical staining for guanase (a, d), immunohistochemical staining for nedasin (b, e), and H&E staining (c, f) in the human normal kidney. Guanase activity was observed in the proximal tubule, but scarcely in the glomeruli, Bowman's capsule, and distal tubule. However, in the immunohistochemical staining for nedasin, a brown discoloration due to DAB oxidation was seen in the proximal tubule but in the distal a little, and not in the glomeruli and Bowman's capsule ; thus, the results differ between the histochemical staining for guanase and the immunohistochemical staining for nedasin.

Figure 3 shows the images of histochemical staining for guanase (a, d), immunohistochemical staining for nedasin (b, e), and H&E staining (c, f) in the human normal small intestine. Guanase activity was observed in the mucosal epithelium(especially goblet cells and apical border), but was not observed in other regions. In the immunohistochemical staining for nedasin, a brown discoloration due to DAB oxidation was observed in the mucosal epithelium (especially goblet cells and apical border), but was not observed in other regions ; thus, consistent results were obtained between the histochemical staining for guanase and the immunohistochemical staining for nedasin.

2. Histochemical staining for guanase and immunohistochemical staining for nedasin in each tissue

Similar results were obtained after staining the liver and kidney 8 times and the small intestine 4 times under the same case. The results of histochemical staining for guanase and immunohistochemical staining for nedasin in the liver, kidney, and small intestine are given (Table 1).

In the liver and small intestine, the results were consistent between the histochemical staining for

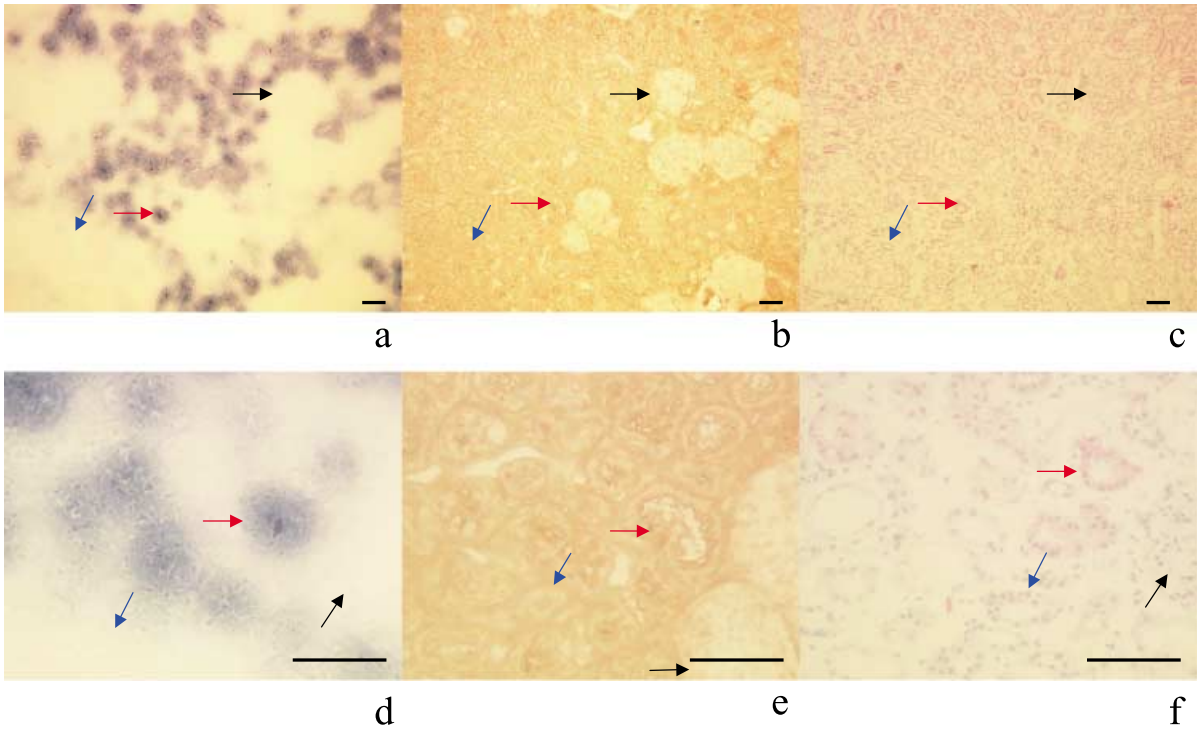

Figure 2 : Histochemical staining for guanase on the frozen sections of the human normal kidney(a: ×100, d: ×400) : Guanase activity was observed in the proximal tubule, but scarcely in the glomeruli, Bowman's capsule, and distal tubule.
Immunohistochemical staining for nedasin on the adjacent sections(b: ×100, e: ×400) : a brown discoloration due to DAB oxidation was seen in the proximal tubule but in the distal tubule a little, and not in the glomeruli and Bowman's capsule.
H&E staining on the adjacent sections(c: ×100, f: ×400).

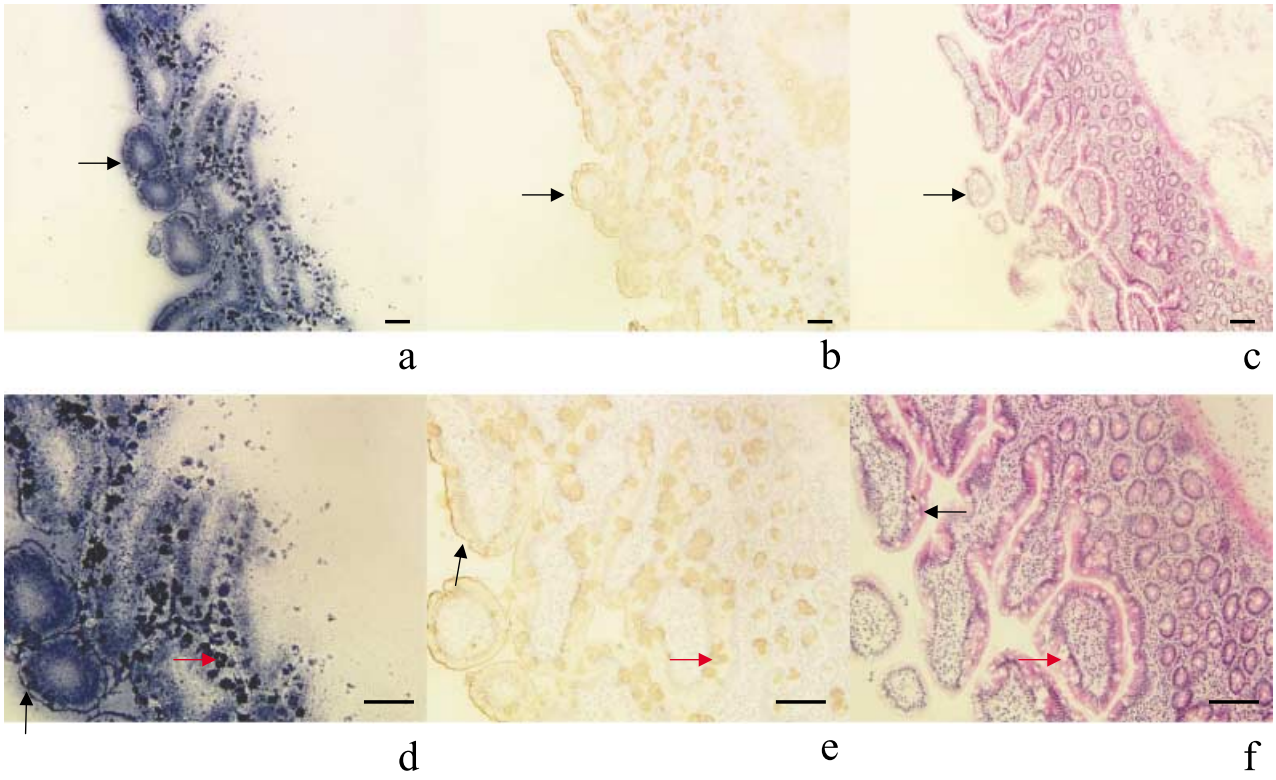

Figure 3 : Histochemical staining for guanase on the frozen sections of the human normal small intestine(a : ×100, d: ×200) : Guanase activity was observed in the mucosal epithelium(especially goblet cells and apical border), and not in other regions. Immunohistochemical staining for nedasin on the adjacent sections(b : ×100, e: ×200) : a brown discoloration due to DAB oxidation was seen in the mucosal epithelium(especially goblet cells and apical border), and not in other regions. H&E staining on the adjacent sections(c: ×100, f: ×200).

guanase and the immunohistochemical staining for nedasin. In the kidney, the results were consistent between the histochemical staining for guanase and the immunohistochemical staining for nedasin in the proximal tubule, glomeruli, and Bowman's capsule, while the results were inconsistent between the histochemical staining for guanase and the immunohistochemical staining for nedasin in the distal tubule.

On the frozen sections of the human striated muscle used in the control experiment, no guanase activity was observed in the histochemical

Table 1. Histochemical staining for guanase and immunohistochemical staining for nedasin in the liver, kidney, and small intestine

		Histochemical staining for guanase	Immunohistochemical staining for nedasin	
Liver	Cytoplasm of hepatocytes	++	++	
	Portal components	−	−	
	Biliary epithelium	+	++	
Kidney	Proximal tubule	++	++	
	Distal tubule	−	+	※
	Glomerulus, Bowman's capsule	−	−	
Small intestine	Mucosal epithelium	+	+	
	goblet cell, apical border	++	++	
Control	Skeletal muscle	−	−	

※ : Inconsistency between the histochemical staining for guanase and the immunohistochemical staining for nedasin
(− : no staining, + : mild staining, ++ : strong staining)

staining as previously reported. When immunohistochemical staining for nedasin was performed in the same manner, a brown discoloration due to DAB oxidation was not observed, but the main samples, including the liver, kidney, and small intestine tissues, were demonstrated to be stained.

DISCUSSION

Guanase is an enzyme that degrades guanine into xanthine and ammonia ; then, the xanthine is degraded into uric acid by xanthine oxidase. As for histochemical studies on guanase, Ito *et al*. (9) reported an investigation of the tissue distribution in a human normal case ; Norstrand *et al*. (15) in the human central nervous system ; Palezki (16) in mouse and rat brain ; Akum *et al*. (17) in the rat central nervous system. Akum *et al*. (17)reported that guanase regulated the morphogenesis of dendrites and formed microtubule assembly.

In reference to a report concerning the DNA sequence of the guanase gene cloned by Yuan *et al*. (13) and a report by Kuwahara *et al*. (14) that an unknown protein, nedasin, which binds to NE-dlg, one of the MAGUK family proteins that are considered to have various important functions in the adhesion of epithelial cells and synaptic connection between neurons, was cloned, whose sequence was identical with that of guanase, immunohistochemical staining for nedasin was performed using a CSA system along with histochemical staining for guanase reported by Ito *et al*. (9) in the human normal liver, kidney, and small intestine. This compared the similarities and differences between the histochemical staining for guanase and immunohistochemical staining for nedasin in each organ.

In the frozen sections of the human normal liver and small intestine, the regions with guanase activity in the histochemical staining and the regions with a brown discoloration due to DAB oxidation in the immunohistochemical staining for nedasin almost overlapped. However, in the previous report by Ito *et al*. (10-12) the stained regions by immunohistochemical staining with anti-human guanase antibody and the stained regions by histochemical staining for guanase, prepared with the human liver, almost overlapped with those of the human kidney. In this study, however, the stained regions by immunohistochemical staining with anti-nedasin antibody and the stained regions by histochemical staining for guanase did not necessarily overlap on the frozen sections of the human normal kidney.

According to the report by Kuwahara *et al*. (14), nedasin was confirmed by northern blot analysis in the human brain, liver, kidneys, and placenta, but not in the skeletal muscle. In addition, there are 4 variants of nedasin, S, V1, V2, and V3 forms, which are organ-specific. More specifically, S and V1 forms are observed in various normal tissues and cancer cells, while V1 and V2 forms are abundant, and S and V3 forms are relatively scarce in the liver. There is no report concerning the 4 variants in the human kidney, but it was suggested that the difference between the regions with guanase activity in the histochemical staining and the regions with a brown discoloration due to DAB oxidation in the immunohistochemical staining for nedasin in the frozen sections of the human normal liver, small intestine and human normal kidney, observed in this study, may be due to the variants of nedasin present in the kidney.

The physiological significance of guanase is poorly understood. The regions where guanase activity was observed are considered to be involved in the absorption of glucose, amino acids, fat, electrolytes, and others in the kidney and small intestine, and also in the transport of various substances in the liver. Since the histochemical staining for guanase and the immunohistochemical staining for nedasin overlapped in many regions, guanase is also presumed to be involved in some signal transduction in these organs like nedasin. Therefore, further comparison between the histochemical staining for guanase and the immunohistochemical staining for nedasin is considered to help the investigation of the physiological significance of guanase ; thus, further studies are needed in the future.

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